Award Number: DAMD17-98-1-8046

TITLE: Identification of the Receptor of the Wnt-1 Signaling Molecule

PRINCIPAL INVESTIGATOR: Raymond Habas, Ph.D.
Xi He, Ph.D.

CONTRACTING ORGANIZATION: Childrens Hospital
Boston, Massachusetts 02115

REPORT DATE: May 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2001	Annual Summary (6 Apr 2000 - 5 Apr 2001)

4. TITLE AND SUBTITLE
Identification of the Receptor of the Wnt-1 Signaling Molecule

5. FUNDING NUMBERS
DAMD17-98-1-8046

6. AUTHOR(S)
Raymond Habas, Ph.D.
Xi He, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Childrens Hospital
Boston, Massachusetts 02115

E-MAIL: raymond.habas@tch.harvard.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color graphics

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The Wnt ligands play crucial roles in mammary tumorigenesis and embryonic development. The *frizzled* family of receptors has been shown to be capable of transducing the Wnt signal suggesting that a *frizzled* protein may encode for the receptor for Wnt-1. In experiments to determine the receptor for Wnt-1, two *frizzled* proteins have been elucidated which may serve as the Wnt-1 receptor suggesting redundancy in this signalling cascade.

Experiments to attempt to identify molecules that interact with dishevelled, a downstream effector, in an effort to understand the transduction of the Wnt signaling has yielded a novel component termed Daam-1. Daam-1 provides a crucial link in the Wnt-1 signal cascade which activate the small GTPase RhoA to mediate cytoskeletal changes and a fundamental link to the non-canonical Wnt pathway termed the Planar Polarity Pathway. Interference with Daam1 function during Xenopus embryogenesis blocks Wnt/PCP but not Wnt/β-catenin signaling, and inhibits morphogenetic cell movements.

These studies suggest Wnt-1 function may be mediated by Rho activation in addition to β-catenin and indeed, Wnt-1 cell transformation is always accompanied by cell shape changes that are often associated with the Rho function. Daam-1 function may provide an important mechanism by which Wnt-1 mediates mammary transformation and tumorigenesis.

Breast Cancer, WNT-1 Signaling Molecule, Receptor

15. NUMBER OF PAGES 13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Date 4th MAY 2001

Table of Contents

Introduction

The Wnt gene family encodes secreted signaling molecules that play important roles in mammary tumorigenesis and embryonic development. The mechanisms of Wnt-1 signaling can provide insights into the molecular nature of mammary tumor development and early embryonic pattern formation.

Our current understanding of the Wnt signal transduction cascade proposes that a member of the *frizzled* (*Fz*) proteins may function as the receptor of the Wnt-1 ligands. I have screened pools containing identified *Fz* molecules using the established *Xenopus* axis duplication system (He et al., 1997) to determine if such a *Fz* molecule can be identified as the receptor for Wnt-1. Furthermore I have screened for interacting molecules for the *Fz* and dishevelled (Dvl) proteins using the yeast two- hybrid system to try and elucidate the biochemical basis of the Wnt signal transduction cascade. The body of this final report summarizes the characterization of a novel clone, Daam-1, identified as a Dvl interacting protein which links Wnt-1 signaling to the small Rho GTPases.

The identification of Daam-1 and elucidation of its function provides novel insights into how Wnt-1 may mediate mammary tumor formation.

BODY

Major Project covering Task 1 in Statement of Work (Months 1-36)

As reported in my previous annual summary, I have used the Xenopus secondary axis duplication assay to screen for *Fz* molecules that may function as the receptor for Wnt-1. With this strategy, I was able to identify from pools of *Fz* molecules, two *fz* proteins that can in the presence of suboptimal concentration of Wnt-CD8 transduce this signal, namely *dFz2* and *mFz8*.

This finding has prompted the conclusion that the Xenopus secondary axis duplication assay may not be able to resolve the finer aspects of interaction of the Wnt-1 protein with a specific *Fz* protein. Thus I am unable to distinguish which one of the proteins may be the true receptor for this ligand. This nature of the true receptor must now rely on a combination of biochemical binding assays with the results from the secondary axis duplication assay. These experiments as summarized in my last annual report are technically difficult due to the inability to produce soluble Wnt-1 protein and such experiments were therefore not attempted.

In summary therefore, my data therefore demonstrates that two *fz* proteins may mediate the secondary axis duplication in the presence of Wnt-1 and this suggests that

more than one *Fz* protein may serve as a receptor for this molecule. This finding may be a direct reflection of the recent finding that the *Fz* and *dFz2* proteins may serve redundant functions in mediating the effects of *wg* (Bhat, 1999 and Kennerdell and Carthew, 1998).

Minor project not outlined in Statement of Work

A second project that I have addressed involved the attempt to find molecules that may transduce the Wnt signal downstream of the *fz* receptor. This project involved a screen for molecule(s) that interact with the dishevelled protein, a downstream molecule genetically and biochemically linked to the signal cascade. The rationale behind this screen was to potentially find a molecule that binds to Dvl that could mediate the Wnt signal cascade allowing me to work back up to the receptor level. To accomplish this screen, two independent fragments of the Dvl protein, mouse dishevelled 2 were used as baits, the DIX domain and the PDZ domain. The screen with the DIX domain as reported in my previous summary yielded no interesting positive clones and I have concentrated on a clone identified with the PDZ domain screen called Daam-1. The positive clones identified in this screen are summarized in table 1

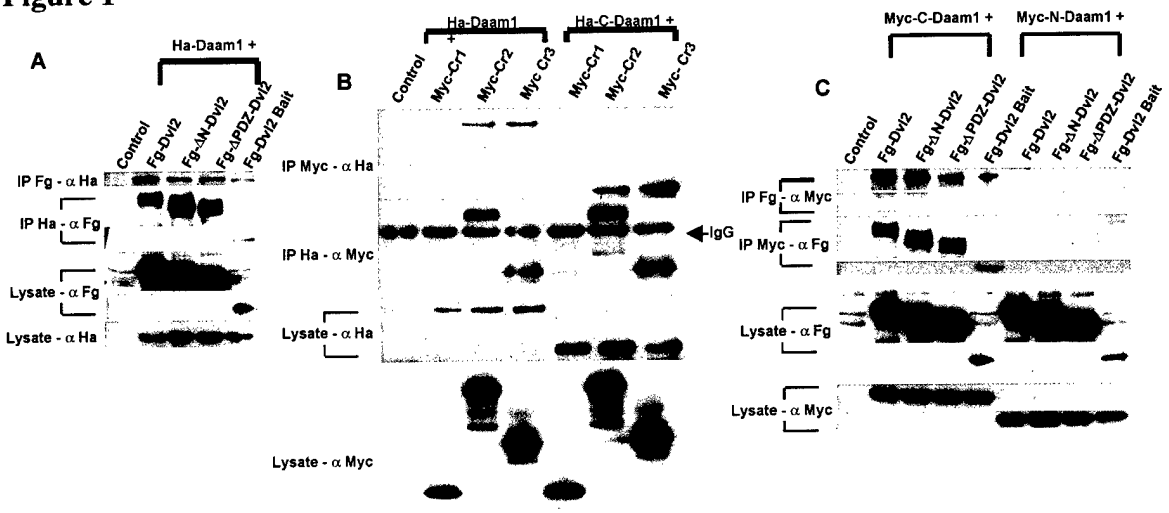
Table 1

#of clones	Identity
18	novel gene called HeXi
2	novel gene called Daam-1
13	cytochrome oxidase
1	nuclear cyclophillin
2	DNA repair enzyme
2	KU autoantigen
1	filamin A
3	dynein
1	HSP40
11	could not rescue

Two independent partial cDNAs were identified from this screen that encoded overlapping carboxyl terminal fragments of a novel protein, referred to as Daam1 (Dishevelled associated activator of morphogenesis). The human Daam1 is widely expressed in various tissues (data not shown) and is a new member of a large family of FH proteins that have been implicated in cell polarity from yeast to human (Wasserman, 1998). The Daam-1 protein contains a central proline-rich FH1 domain and a more carboxyl FH2 domain. The Daam-1 proteins share a unique but conserved carboxyl-terminal region that spans the two Daam1 fragments from the two-hybrid screen,

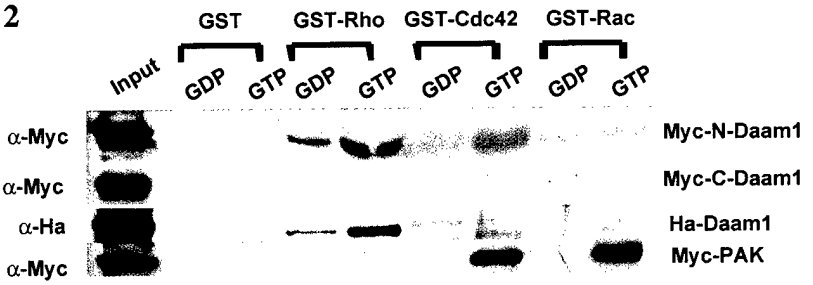
potentially defining a region mediating Dvl-Daam1 interaction. This interaction was confirmed by co-immunoprecipitation assays and it was verified that Daam-1 interacted with Dvl through its carboxyl tail. Interestingly it was observed that C-Daam-1 interacted with two domains of Dvl, the central PDZ and C-terminal DEP domains (Figure 1 A, B and C).

Figure 1



The amino terminal half of the Daam-1 protein is conserved but distinct from other FH proteins (data not shown), and by analogy, may represent a binding domain for Rho, Rac or Cdc42 (Watanabe et al., 1997). This hypothesis was tested using fusion proteins of Rho, Rac or Cdc42 preloaded with GTP or GDP. As shown in Figure 2, the N-terminus of Daam-1 preferentially interacts with GTP loaded Rho but not Rac or Cdc42. Pak was used as a positive control for interaction with Rac or Cdc42.

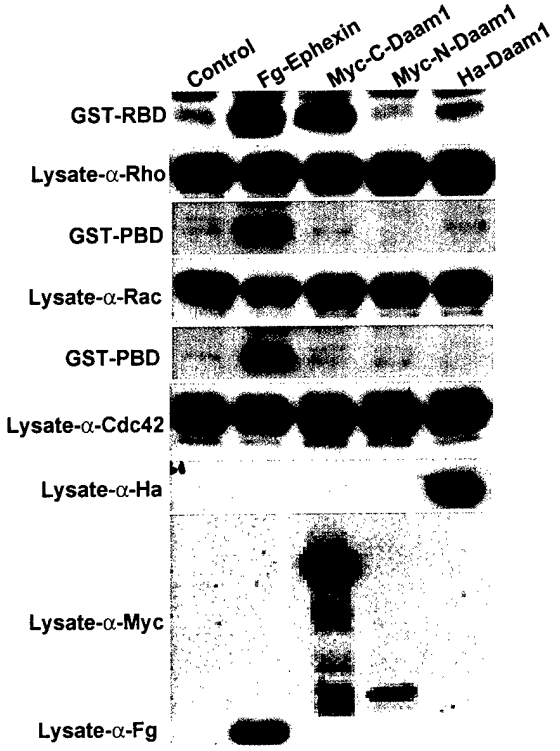
Figure 2



As Daam-1 interacted with the Rho GTPase, I examined whether Damm-1 can mediate Rho Rac or Cdc42 activation. This is performed using a method described by (Ren et al., 1999 and Benard et al., 1999) which involved using a GST-RBD or GST-PDB fusion protein which recognizes the activated form of Rho or Rac/Cdc42 respectively. HEK 293T cells were transfected with full length Daam-1, N-terminal fragment (N-Daam-1) or C-terminal fragment (C-Daam-1). These experiments

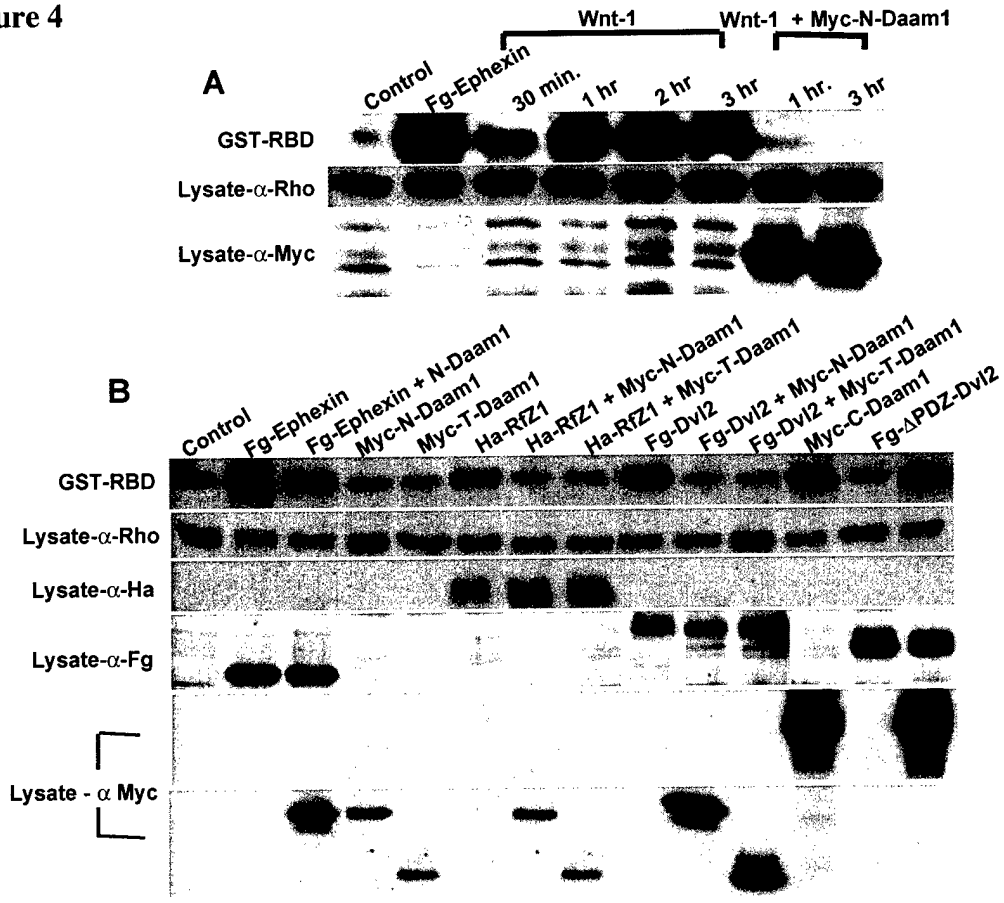
demonstrated that C-Daam-1 expression led to robust Rho activation mimicking the positive control GEF, Ephexin (Shamah et al., 2001). Strikingly no Rac or Cdc42 activation was seen by any of the Daam-1 constructs (figure 3).

Figure 3



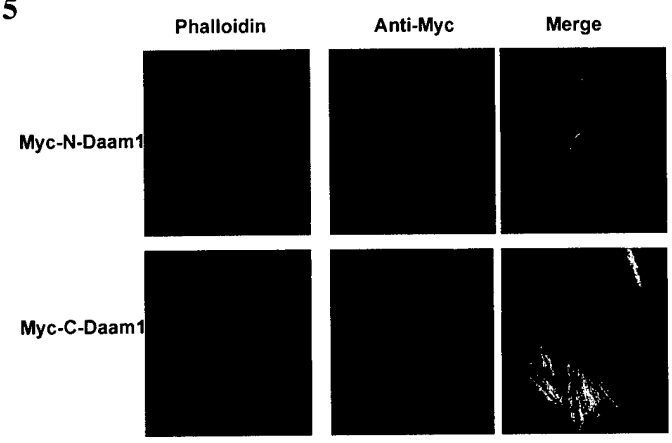
As Daam-1 was capable of triggering Rho activation, I tested whether Wnt-1 can induce Rho activation and also as the N-Daam-1 construct was incapable of activation Rho, it was tested whether this construct can interfere with Rho activation. The results of these experiments are shown in Figure 4, in which it was demonstrated that Wnt-1 could strikingly induce Rho activation in a time dependent manner (figure 4A). This activation was not inhibited by cycloheximide which blocks new protein synthesis suggesting that Wnt-1 triggers Rho activation directly (data not shown). It was also observed that *Fz* and Dvl can induce Rho activation which is blocked by N-Daam-1 demonstrating that the Wnt induction of Rho functioned through Fz, Dvl and Daam-1 (Figure 4A and B). Notably removal of the PDZ domain from Dvl abrogates Rho activation and can function as a dominant negative construct for Wnt, *Fz* or Dvl mediated Rho activation (data not shown) and this is the region that was used in the two-hybrid screen that identified Daam-1. N-Daam-1 or T-Daam-1, the rat fragment isolated from the two-hybrid screen, does not block Rho activation induced by Ephexin suggesting that the effects of N-Daam-1 are specific for the Wnt/*Fz*/Dvl cascade (Figure 4A and B).

Figure 4



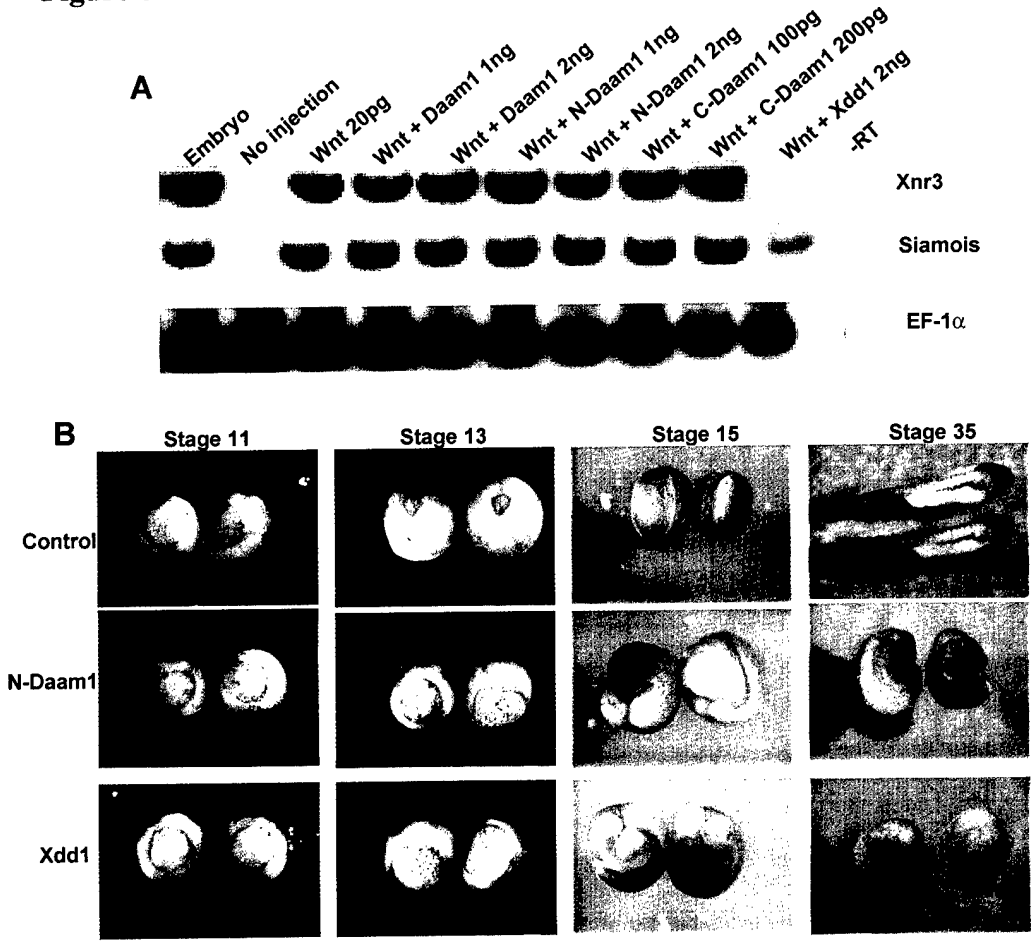
As Daam-1 is linked to the Rho pathway, it was tested whether the dominant-negative (N-Daam-1) or activated (C-Daam-1) constructs exerted effects on the actin cytoskeleton. Transfection of these constructs into Hela cells resulted in striking effects on actin stress fiber formation and the actin cytoskeleton. N-Daam-1 induced a marked collapse of the actin cytoskeleton whereas C-Daam-1 induced the formation of numerous parallel stress fibers (figure 5), a hallmark of Rho activity.

Figure 5



As Daam-1 couples Wnt signaling to the Rho cascade, a pathway identified genetically in Drosophila termed the Planar Cell Polarity Pathway (Mlodzik, 1999), and in Xenopus this pathway is associated with cell migratory movements during gastrulation (Sokol, 2000), I tested the effects of Daam-1 in developing Xenopus embryos. The Xenopus homologue of Daam-1 is ubiquitously expressed (data not shown) and expression of the previous Daam-1 constructs did not affect the canonical Wnt/β-catenin pathway as judged by the inability of these constructs to interfere with XWnt-8 induction of Siamois and Xnr-3 (Figure 6A). However expression of N-Daam-1 dorsally resulted in a severe failure of cellular migration for gastrulation (Figure 6B). This phenotype is similar to the observed phenotypes observed with expression of dominant-negative constructs of Dsh, Xwnt-11 or XfZ7, all of which are implicated in this pathway.

Figure 6



In data not shown, the dominant-negative N-Daam-1 construct blocks convergent extension movements of explanted animal poles treated with activin and the activated C-

Daam-1 construct is able to rescue the block of convergent extension movements induced by dominant-negative constructs of Xwnt-11, XfZ7 and Xdsh. These data suggest that the Daam-1 protein functions in the Wnt pathway mediating gastrulating cell movements.

Summary

The experiments performed thus far encompassed the project outlined in the Statement of Work for months 1-36. The data presented summarizes the continued inability of the initial proposed experimental approach to identify a single receptor for the Wnt-1 molecule. The data from these experiments however do strongly suggest that Wnt-1 may be able to mediate its signaling abilities by interacting with more than one *Fz* molecule. Concurrent with these finding are the published data from Drosophila demonstrating redundancy in the roles of the two *Fz* molecules in mediating *Wg* signaling.

The use of the yeast two-hybrid system, with the PDZ domain of Dvl as a bait was successful at identifying a novel molecule which links Wnt-1 signaling to the small Rho GTPase, RhoA. Daam-1 analysis provides some useful clues into this cascade that mediates the genetically identified Planar polarity Pathway. Whilst the genetic screens in Drosophila has identified components of this pathway namely *Fz*, Dvl and Rho, there has been no evidence of a biochemical activation of Rho by any of these components. I have demonstrated that Wnt-1, *Fz* and Dvl through the novel protein, Daam-1, leads to the biochemical activation of RhoA. I have shown that Daam-1 has profound effects on the actin cytoskeleton implicating that Wnt-1 may induce cytoskeletal changes for cellular transformation through the Daam-1 protein. I have presented evidence also that this pathway is functional in early Xenopus development affecting gastrulating cell movements indicating that this novel β-catenin independent pathway plays critical roles in cytoskeletal functions for migrating cells.

The ability of Wnt-1 to biochemically trigger the activation Rho may help to explain its ability of Wnt-1 to induce cell shape changes observed in mammary transformation and provide novel insights into this transformation process. Interestingly also this effect can be uncoupled from the cell fate and proliferation induced through the Wnt/β-catenin pathway suggesting that the Wnt/Daam-1 pathway may exert effects directly on the cellular actin cytoskeleton.

In summary the continued efforts at understanding the mechanisms of Wnt-1 signal transduction such the analysis of the Daam-1/Rho cascade can help to elucidate the complex role of this signaling molecule in cellular transformation and embryonic development. The identification and continued studies of Daam-1 can help to provide

valuable insights into the ability of Wnt-1 to mediate cellular changes observed in mammary tumorigenesis.

Key research accomplishments

- identified two frizzled molecules that can transduce Wnt-1 signaling.
- identified a novel Dvl-binding molecule, Daam-1.
- demonstrated that Daam-1 is involved in RhoA activation and is required for Wnt-1, *Fz* and Dvl mediated biochemical activation of RhoA.
- demonstrated that Daam-1 functions in early Xenopus embryonic development mediating gastrulation cell movements implicating RhoA as a major component of this pathway.

Reportable Outcomes

- none

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